# Comparative Hydrolytic Approaches Using Bacterial Cellulases for Valorization of Pomegranate Peels Waste

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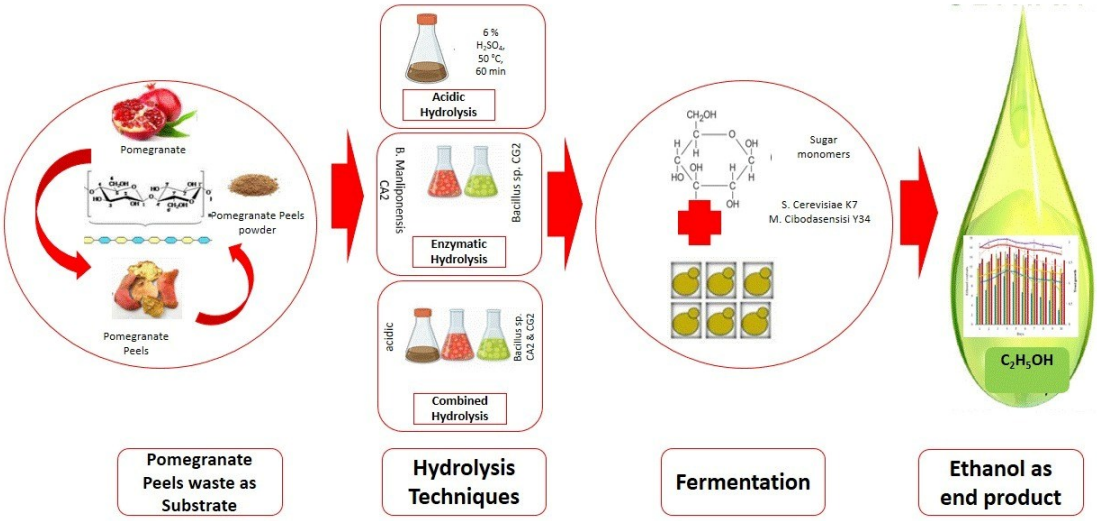
## GRAPHICAL ABSTRACT



# Comparative Hydrolytic Approaches Using Bacterial Cellulases for Valorization of Pomegranate Peels Waste

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The sustainable management of fruit waste offers both economic and environmental benefits, particularly in addressing the global energy crisis. This study explores the conversion of pomegranate peel waste (PPW) into bioethanol through acidic, enzymatic, and combined (acidic + enzymatic) hydrolysis followed by fermentation. Cellulolytic bacterial isolates *Bacillus sp.* CG2 and *Bacillus manliponensis* CA2 were used to perform enzymatic and combined hydrolysis of the PPW with cellulolytic potential of 0.27±0.011 and 0.265±0.05 IU, respectively. Enzymatic hydrolysis was performed at 50 °C with PPW, enzyme dosage, and acetate buffer for different time intervals while optimum conditions for acidic saccharified PPW were 6% $H_2SO_4$, 50 °C, 60 min. The combined hydrolysis approach used a 1:1 ratio of acidic and enzymatic hydrolysates, yielding maximum reducing and total sugar concentrations of 85.77±1.21 g/L and 179.18±3.42 g/L, respectively, after 96 h. *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34, was employed to ferment treated PPW hydrolysates. *M. cibodasensis* Y34 in combined hydrolysate produced the highest ethanol concentration (16.05 ± 0.66 g/L), with a fermentation efficiency of 83.8% and ethanol yield of 0.41 g/g. These findings highlighted the potential of integrated hydrolysis techniques for efficient bioethanol production from fruit waste.

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## INTRODUCTION

The growing global need for sustainable bioresources and eco-friendly waste management strategies has intensified interest in converting agro-industrial residues into valuable products. Among these residues, pomegranate peels (*Punica granatum* L.), which are frequently thrown away during juice extraction and processing activities, pose a considerable environmental challenge. However, they are rich in lignocellulosic materials

and polyphenolic compounds, rendering them potential feedstocks for value addition through fermentation (Demiray *et al*. 2020; Dhande *et al*. 2021; Chaudhary *et al*. 2025). Pomegranate (*Punica granatum*) is a fruit-bearing shrub from the Lythraceae family and is cultivated widely from its ancient origins in Iran to South and Central Asia, the Middle East, Africa, and the Mediterranean Basin (Mo *et al*. 2022). It holds considerable cultural, nutritional, and economic significance across the globe.

On the global agricultural stage, annual pomegranate production is estimated at around 3 million tonnes (Renjini 2024). Pakistan Produces roughly 57.8 thousand tonnes of pomegranates annually contributing significantly to the waste produced by the processing sector. Every year, about 1.9 million metric tonnes of pomegranate peels are produced worldwide (Khursheed *et al*. 2025). The pomegranate juice and processing industries produce massive amounts of agro-industrial by-products, particularly pomegranate peel waste (PPW). Globally, pomegranate processing yields approximately 500 to 550 kg of pomace waste per tonne of fresh fruit. Pomegranate comprising of 75% to 80% of pomace, 26% to 30% peels, whereas the peels typically contain 70% to 75% moisture (Maggiore and Setti 2025). Moreover, on a global scale, this implies approximately 1.62 million tonnes of waste annually (Ko *et al*. 2021). Lignocellulosic biomass, which mainly consists of cellulose, hemicellulose, and lignin, poses challenges in efficient hydrolysis due to its compact structure. Traditional acid hydrolysis, primarily breaking down hemicellulose and lignin, can produce fermentation inhibitors such as furfural and 5-hydroxymethylfurfural, conceding downstream bioconversion processes (Straathof 2023). In contrast, enzymatic hydrolysis employing cellulases offers a minor alternative, which in return yield some inhibitory compounds and fermentable sugars (Chen *et al*. 2025).

According to recent research, bacterial cellulases are superior biocatalysts as compared to their fungal counterparts. Bacterial enzymes exhibit better thermostability, wide pH range, and greater substrate specificity, required for expedient industrial bioprocessing (Agrawal and Verma 2021; Hosny and El-Sheshtawy 2022). Cellulosome complexes from thermophilic and cellulolytic bacteria exhibit efficiency in breaking down crystalline cellulose (Hu and Zhu 2019). *Bacillus* species are well known for their strong cellulolytic potential. The genus *Bacillus* has been investigated for biofuel production due to their capacity to use complex substrates and withstand a variety of environmental conditions. They can produce a variety of hydrolytic enzymes, such as endoglucanases, exoglucanases, and β-glucosidases, which are crucial for the breakdown of lignocellulosic biomass (Chukwuma *et al*. 2025; Choińska-Pulit *et al*. 2025). Over the past decades, numerous *Bacillus* species possessing cellulolytic potential *viz*. *Bacillus subtilis*, *B. licheniformis*, *B. methylotrophicus*, *B. velezensis*, *B. manliponensis*, and *B. megaterium*, have been screened and characterized *(*Ma *et al*. 2020; Munir *et al*. 2021; Shehzadi *et al*. 2024). *B. subtilis* from rotten wood and forest soil, *B. velezensis* from waste water*, B. manliponensis* from rotten fruit, and *B. megaterium* from fermented wheat straw possesses robust cellulase enzyme secretory systems (Tasaki *et al*. 2017). From literature, the reported CMCase activities were 0.22 to 2.28 U/mL by *Paenibacillus lactis*, *Lysinibacillus macroides*, *Lysinibacillus fusiformis*, and *B. cereus*, 0.9 µmol/mL/min by *Paenibacillus sp.*, 0.27±0.011 and 0.265±0.05 IU by *Bacillus sp.* CG2 and *B. manliponensis* CA2 (Islam *et al*. 2018; Ali *et al*. 2019; Shehzadi *et al*. 2024). Furthermore, the combined application of acid and enzymatic hydrolysis has been reported to release sugars synergistically by disrupting lignin barriers and facilitating enzymatic access to cellulose (Wu *et al*. 2023). However, the majority of research concerning the valorization of pomegranate peel has

concentrated on acid hydrolysis exclusively or on fermentation techniques involving the entire biomass with chemical pretreatment (Chaudhary *et al*. 2021; Saleem *et al*. 2022). There has been a lack of studies that directly compare various hydrolytic methods utilizing bacterial cellulases on pomegranate peels. Moreover, most studies highlight fungal cellulases or commercial enzymes, ignoring bacterial enzyme diversity (Srivastava *et al*. 2018).

The present study bridges this gap by evaluating the comparative hydrolysis strategy employing two bacterial cellulolytic strains (*Bacillus sp.*. CG2 and *Bacillus manliponensis* CA2 with cellulolytic potential values of 0.27±0.011 and 0.265±0.05 IU) under three hydrolytic treatments *viz*., acidic, enzymatic, and combined on PPW. The ability of *Bacillus* species to grow on cellulosic substances and production of cellulase enzymes suggests its potential role in lignocellulose breakdown and sustainable biomass use. The addition of *B. manliponensis* CA2 and *Bacillus sp.* CG2 in cellulolytic research provides valuable information about novel bacterial sources for efficient cellulose degradation and bioethanol production. The integration of pretreatments has the potential to achieve synergistic effects, optimizing release of reducing sugars, altering the lignocellulosic composition, improving bioethanol titer, yield, and fermentation efficacy. By comparing these hydrolytic strategies, this study aims to advance a more sustainable and economically viable biorefinery model for fruit waste valorization thus contributing to circular bioeconomy approaches.

## MATERIALS AND METHODS

### Collection of Substrates from Various Regions

In present In order to obtain the pomegranate peels waste (PPW) as a source of substrates in the present study, various areas of Lahore and also the Narowal were selected for substrate collection. Fresh peels were collected from October to November (peak availability time in Pakistan). Temperature (25 to 30 °C) and humidity (60 to 70%) was moderate. After collection, peels were washed with distilled water. The PPW were dried at 60 °C in an oven until they reached a consistent weight. With help of an electrical grinder, peels were ground into powdered form to obtain particles smaller than 1 mm using a sieve.

### Constituents' Extraction and Analysis of PPW

The dried PPW (1.0 g) were homogenized in 100 mL of the distilled water and shaken in an incubator at 37 °C and 200 rpm overnight, followed by centrifugation (5000 rpm, 4 °C). The filtrate was used for assessment of total reducing (DNS method, Miller 1959) and total sugar (phenol sulfuric acid method, Dubois *et al*. 1956). The assessment with 3,5-dinitrosalicylic acid reagent, 1 mL of filterate was mixed with 3 mL reagent, boiled for 5 min, cooled, and absorbance was measured at 540 nm. Similarly, 1 mL of diluted filterate was treated with 0.5 mL 5% aqueous phenol solution followed by 2.5 mL concentrated sulfuric acid. Absorbance was recorded at 492 nm. Dried PPW were utilized to measure the cellulosic contents *viz*., extractives, hemicellulose, lignin, and cellulose (Lin *et al*. 2010). The dried peels (10%) were first heated in ethanol at 100 °C for 5 min to remove extractives followed by the boiling of the residue (dried at 60 °C) with 0.5 M NaOH (100 mL) for 15 minutes to extract hemicellulose. The remaining residue was treated sequentially (10 mL) with 1.25 and 72% sulfuric acid for two hours to quantify lignin content (subtracting the values of both acid treatment). Cellulose content was calculated

by subtracting the combined percentages of extractives, hemicellulose, and lignin from 100.

## Microbe Selection for Study

Bacteria and yeast isolates having cellulolytic and ethanologenic potentials were procured from the Microbiology research laboratory of Department of Zoology, University of Education, Lahore, Pakistan. The bacterial isolates used were *Bacillus sp.* CG2 (accession No. OM974175) and *Bacillus manliponensis* CA2 (accession No. ON324120) possessing cellulytic potential 0.265±0.05 and 0.27±0.011 IU (Shehzadi *et al*. 2024). For purposes of comparison, sugar contents *cibodasensis* Y34 and *Saccharomyces cerevisiae* K7 served as experimental and standard yeast for the study (Chaudhary and Karita 2017).

## Hydrolysis/Saccharification Experiment

Three different forms of Saccharification *viz*., acidic, enzymatic, and combined (acidic+enzymatic) were carried out by applying PPW as a substrate source.

## Acidic Saccharification/ Hydrolysis

The optimized conditions (6% v/v sulfuric acid, 50 ℃, 60 min) for acidic hydrolysis was adopted from the previous study (Saleem *et al*. 2022). For acidic hydrolysate, powdered peels and dilute acid ratio of 1:10 w/v was adopted. Hydrolysis was performed for 60 min in water bath at a temperature of 50 ℃. After completing the filtration process *via* vacuum filtration assembly, the filtrate was treated with 2.5% charcoal (w/v) and agitated for one hour with 200 rpm at 37 ℃ to detoxify the phenolic by-products produced by acid (Costa-Trigo *et al*. 2020). Acid treatment of the substrate produced furfural and phenolic compounds that inhibit the fermentative efficiency of yeast. Detoxified hydrolyzate was filtered again. The estimation of total phenolics in the hydrolysate was performed using the Folin Ciocalteu method, as outlined by Gonzalez *et al*. (2003). The neutralization of the filtrate (acid hydrolyzate) was performed by adding pellets of NaOH up to pH 5 matching the conditions used in enzymatic saccharification.

## Enzymatic Saccharification/Hydrolysis

In order to perform the enzymatic hydrolysis, substrate buffer and crude enzyme were prepared. For substrate buffer powdered PPW and acetate buffer was taken in a ratio of 1:10 w/v. Acetate buffer, 0.2 M for a pH of 5, was prepared. In sterilized substrate buffer (100 mL), crude enzyme dose v/v (0.27 IU) for both enzymes were inoculated, and were placed for about 5 days in a water bath at 50 ℃. Samples were drawn after 4, 8, 12, 16, 20, 24, 48, 72, 96, 120 hours. Samples were then subjected to estimation of different contents *i.e.*, reducing and total sugars (Dubois *et al*. 1956; Miller 1959). Percent Saccharification (Alrumman 2016) was computed by following Eq. 1:

$$\% \text{ Saccharification} = [(\text{Reducing sugars released} \times 0.9) / (\text{Initial substrate conc.})] \times 100 \quad (1)$$

Crude enzyme was prepared in basal medium (pH 7.0) comprising 0.1 g of yeast extract, 0.01 g of the magnesium sulphate, 0.7g of the disodium hydrogen phosphate, 0.2 g of the potassium dihydrogen phosphate, 0.01 g of the sodium citrate and for carbon source 1.0 g of carboxy methyl cellulose (CMC) in 100 mL of distilled water (Bai *et al*. 2012). In autoclaved medium, both cellulose degrading bacteria (1.0 mL) were inoculated separately and incubated at 37 ºC for 72 h in a shaking incubator at 200 rpm (Abu-Gharbia *et al*. 2018).

## Combined Saccharification/Hydrolysis

Combined hydrolysis involves the combination of the acidic as well as enzymatic hydrolysis. To perform combined hydrolysis, acid hydrolyzate was prepared first with 6% w/v sulfuric acid at 50 ℃ for about 60 min. Acid hydrolyzate was neutralized (NaOH) to pH 5 and detoxified by charcoal (2.5% w/v). About 50 mL of acid hydrolyzate (peels and acid 1:10 w/v) was mixed with 50 mL acetate buffer and sterilized by autoclaving for 15 minutes. Enzyme dosage of 0.27 IU v/v were used for enzymatic hydrolysis by placing the mixture for 5 days at 50 ℃ in water bath.

Samples were drawn after 4, 8, 12, 16, 20, 24, 48, 72, 96, 120 hours and were subjected for biochemical testing to measure quantitatively the reducing and total sugar contents (Dubois *et al*. 1956; Miller 1959). Percent saccharification was then calculated.

## Fermentation Experiments

*Substrate for fermentation experiment*

Acidic, enzymatic, and combined hydrolyzates served as source of sugars for fermentation experiment. Hydrolyzates were prepared at their optimized conditions.

*Preparation of the synthetic media having the cellulose as a substrate*

To formulate the fermentation medium, hydrolysates were mixed with the synthetic medium in equal proportions (1:1 v/v) (Chaudhary and Karita 2017). The composition of synthetic medium included 2.72 g of $KH_2PO_4$, 2.6 g of $(NH_4)_2SO_4$, 6 g of sodium citrate, 6.5 g of yeast extract, 0.3 g of $CaCl_2$, 20 g of cellulose, 0.00042 g of $ZnCl_2$, 0.8 g of $MgSO_4.7H_2O$, and 1.5 g of citric acid in 1L of distilled water. Fermentation medium was inoculated by 5% v/v yeast inocula (OD of yeast cells set at 0.5 at 600 nm in phosphate buffer saline). Yeast isolates were revived in MYG medium comprising 3 g of the yeast extract, 3 g of the malt extract, 5 g of the peptone, and 10 g of the glucose in 1.0 L of distilled water for 24 hours.

Fermentation was carried out in narrow-necked glass bottles with screw caps to prevent evaporation and autoclaving was used to sterilize the apparatus. The fermentation medium (pH 5) was incubated at 30 ℃ statically in screwed capped bottles. Fermentation experiments continued up to 10 days, and samples were drawn daily for testing of different contents, *i.e.*, reducing sugars, ethanol titer, and yeast growth. By using a spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent Technologies), about 600 nm growth of yeast in fermentation medium was evaluated (Mira *et al*. 2022). Consumed reducing sugars were calculated by substracting initial sugars by final sugars obtained after consumption in fermentation medium.

Ethanol yield and fermentation efficiency were assessed by using Eqs. 2 and 3:

$$\text{Yield of ethanol (g/g)} = \frac{\text{Contents of ethanol } (\frac{g}{L})}{\text{Consumed reducing sugar } (\frac{g}{L})} \quad (2)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Practical ethanol yield}}{\text{Theoretical ethanol yield}} * 100 \quad (3)$$

## Statistical Analysis

All experimentation was performed with triplicates. One-way ANOVA was used to determine statistical significance at $p < 0.05$, followed by Duncan's Multiple Range Test for mean comparison.

## RESULTS

### Selection of Bacterial Isolates

Isolates of bacteria *Bacillus sp.* CG2 (accession No. ON974175) and *Bacillus manliponensis* CA2 (accession No. ON324120) manifested the cellulase (CMCase) activity as 0.27±0.011 and 0.265±0.05 IU on carboxymethyl cellulose (Shehzadi *et al*. 2024). These results confirmed the potential of both isolates for further applications in bioconversion of PPW.

### Comparison of Different Techniques for Hydrolysis of PPW

In the current study, three different hydrolytic techniques *viz.*, acidic, enzymatic and combined (acidic as well as enzymatic) were used.

### Reducing Sugar Estimation

The optimized PPW saccharification conditions from previous study were; Concentration of dilute sulfuric acid 6%, temperature 50 °C and duration of saccharification were 60 minutes (Saleem *et al*. 2022). Using these optimized conditions, 55.45 g/L of reducing sugars were estimated. Maximum reducing sugars were produced under combined hydrolysis (acidic + enzymatic) at 96 h, especially by *Bacillus sp.* CG2 (85.77 ± 1.21 g/L) and *Bacillus manliponensis* CA2 (79.51 ± 2.44 g/L). Enzymatic treatment showed a steady increase over time, peaking at 96 h as well depicting values of 53.29 ± 2.20 g/L, 51.05 ± 1.06 g/L by *Bacillus sp.* CG2 and *Bacillus manliponensis* CA2 correspondingly. The combined hydrolysis boosts sugar release due to synergistic effects of chemical pre-treatment breaking lignin/hemicellulose structure and enzymatic action efficiently hydrolyzing cellulose (Table 1).

**Table 1.** Effect of Different Pretreatment Methods on Reducing Sugars (g/L) from PPW at Different Time Interval

| Time (h) | Non treated PPW | Acidic Treat-ment | Enzymatic Treatment | | Combined Treatment | |
|---|---|---|---|---|---|---|
| | | | CG2 | CA2 | CG2 | CA2 |
| **4** | 22.1 ±0.12 | 55.45 ±1.62 | 23.03±1.27$^{F}$ | 22.80±0.33$^{C}$ | 67.13±1.67$^{c}$ | 59.55±4.19$^{C}$ |
| **8** | | | 25.02±3.69$^{E,F}$ | 24.36±1.39$^{C}$ | 73.03±1.56$^{B,C}$ | 65.23±1.69$^{B,C}$ |
| **12** | | | 29.24±2.28$^{D,E,F}$ | 28.85±2.43$^{B,C}$ | 74.22±2.42$^{B,C}$ | 65.42±0.90$^{B,C}$ |
| **16** | | | 30.00±1.48$^{C,D,E,F}$ | 29.36±0.43$^{B,C}$ | 75.04±1.55$^{B,C}$ | 66.49±2.00$^{B,C}$ |
| **20** | | | 32.12±1.26$^{C,D,E}$ | 31.44±7.96$^{A,B,C}$ | 75.96±0.76$^{B}$ | 68.15±2.48$^{B,C}$ |
| **24** | | | 35.91±0.94$^{C,D}$ | 32.80±5.52$^{A,B,C}$ | 78.14±1.42$^{A,B}$ | 69.1±1.22$^{A,B,C}$ |
| **48** | | | 38.72±1.12$^{B,C}$ | 36.83±9.72$^{A,B,C}$ | 78.08±1.49$^{A,B}$ | 70.53±1.42$^{A,B,C}$ |
| **72** | | | 46.51±3.58$^{A,B}$ | 43.08±3.85$^{A,B}$ | 79.84±2.26$^{A,B}$ | 71.25±0.42$^{A,B}$ |
| **96** | | | 53.29±2.20$^{A}$ | 51.05±1.06$^{A,B}$ | 85.77±1.21$^{A}$ | 79.51±2.44$^{A}$ |
| **120** | | | 47.11±0.14$^{A,B}$ | 45.37±0.89$^{A}$ | 77.23±1.26$^{B}$ | 72.3±0.43$^{A,B}$ |

Data are presented as mean values of triplicates ± standard error of the mean (SEM). Within each column, values not sharing a common letter differ significantly ($P \leq 0.05$) based on one-way ANOVA.

## Total Sugar Evaluation

Estimated total sugars as a result of acidic hydrolysis of PPW on optimized conditions were 122.2 g/L. Same findings were observed for total sugars from enzymatic hydrolysis during which the maximum amount of the total sugars *i.e.*, 119.26±3.19 g/L was documented after 96 h by *Bacillus sp.* CG2, while *Bacillus manliponensis* CA2 produced 117.39±20.48 g/L at the same hours. Sharp decreases (76.28±14.30, 76.86±3.36) in sugars were recorded at 120 hours by both strains *Bacillus sp.* CG2 and *Bacillus manliponensis* CA2, correspondingly.

The large standard error observed in the *Bacillus manliponensis* CA2 hydrolysis at 96 hours might be due to differences in microbial performance across replicates and enzyme activity fluctuation over time. Comparable results were documented after combined hydrolysis performed for total sugars during which a regular rise was detected in the amount of total sugars from 4 h until 96 h followed by a sharp decrease at 120 hours. The measured values were 197.18 ± 3.42 g/L for *Bacillus* sp. CG2 and 192.71 ± 1.20 g/L for *Bacillus manliponensis* CA2. (Table 2). This further validates that combined treatment enhanced total sugar release by maximizing both hemicellulose and cellulose hydrolysis.

**Table 2.** Total Sugar Contents (g/L) from PPW under Different Pretreatment Methods and Time Interval

| Time Period (h) | Non-treated PPW | Acidic Treatment | Enzymatic Treatment | | Combined Treatment | |
|---|---|---|---|---|---|---|
| | | | CG2 | CA2 | CG2 | CA2 |
| **4** | 75.8 ± 0.06 | 122.17± 10.38 | 46.16± 2.98$^{C}$ | 43.81± 10.13$^{C}$ | 149.09± 2.22$^{F}$ | 141.85± 1.41$^{E}$ |
| **8** | | | 48.77± 8.22$^{C}$ | 46.41± 2.94$^{C}$ | 153.35± 2.23$^{F}$ | 147.46± 2.98$^{D, E}$ |
| **12** | | | 57.72± 22.02$^{C}$ | 52.45± 1.16$^{B, C}$ | 158.95± 3.21$^{E,F}$ | 151.92± 3.39$^{C,D,E}$ |
| **16** | | | 62.85± 8.03$^{B,C}$ | 60.90± 2.68$^{A,B,C}$ | 164.44± 1.35$^{D,E,F}$ | 159.96± 9.38$^{B,C,D,E}$ |
| **20** | | | 69.91± 4.65$^{A,B,C}$ | 67.04± 1.04$^{A,B,C}$ | 173.14± 4.73$^{C,D,E}$ | 167.74± 0.86$^{B,C,D}$ |
| **24** | | | 80.92± 3.12$^{A,B,C}$ | 76.68± 4.17$^{A,B,C}$ | 179.99± 2.41$^{B,C.D}$ | 173.83± 6.18$^{A,B,C}$ |
| **48** | | | 89.31± 7.55$^{A,B,C}$ | 87.30± 6.13$^{A,B,C}$ | 186.3± 4.01$^{A,B,C}$ | 180.66± 4.38$^{A,B}$ |
| **72** | | | 111.81± 6.64$^{A,B}$ | 107.64± 27.43$^{A, B}$ | 194.43± 3.96$^{A,B}$ | 189.3± 2.52$^{A}$ |
| **96** | | | 119.26± 3.19$^{A}$ | 117.39± 20.48$^{A}$ | 197.18± 3.42$^{A}$ | 192.71± 1.20$^{A}$ |
| **120** | | | 76.28± 14.30$^{A,B,C}$ | 76.86± 3.36$^{A,B,C}$ | 181.01± 4.02$^{B,C,D}$ | 177.42± 2.68$^{A, B}$ |

Data are presented as mean values of triplicates ± standard error of the mean (SEM). Within each column, values not sharing a common letter differ significantly ($P \leq 0.05$) based on one-way ANOVA.

**Table 3.** Lignocellulosic Contents in Different Hydrolysis Treatments of Pomegranate Peels Waste

| Lignocellu-losic contents (%) | Non Treatment | Acidic | Enzymatic | | Combined | |
|---|---|---|---|---|---|---|
| | | | CG2 | CA2 | CG2 | CA2 |
| **Extractives** | 22.07±0.40 | 17.47±6.49 | 25.00±1.15 | 23.30±1.31 | 31.07±5.59 | 29.33±2.75 |
| **Hemicellulose** | 30.01±1.10 | 19.19±6.63 | 14.63±1.94 | 21.25±1.32 | 13.65±2.74 | 16.35±4.00 |
| **Lignin** | 15.30±1.30 | 14.99±2.11 | 14.82±3.99 | 10.32±1.6 | 12.12±10.44 | 8.30±2.26 |
| **Cellulose** | 32.62±0.40 | 48.35±2.29 | 45.55±.28 | 45.13±1.87 | 43.16±12.41 | 46.02±6.43 |

## Lignocellulosic Contents Estimation

The cellulosic contents of PPW were estimated before and after treatments, as documented in Table 3. Non-treated samples had higher hemicellulose and lignin contents. The cellulose, lignin, and hemicellulose contents were reduced by treatments such as acidic and enzymatic.

In the case of combined hydrolysis, hemicellulose and lignin dropped up to 13.65±2.74% and 12.12±10.44% with *Bacillus sp.* CG2 as well as 16.35±4.00%, 8.30±2.26% with *Bacillus manliponensis* CA2 respectively. Cellulose contents for both strains were recorded as CG2: 43.1±12.41%, CA2: 46.02±6.43%. There was a decrease in hemicellulose (19.19±6.63%) with acidic treatment, whereas enzyme treatment and combined treatment retained more hemicellulose than acidic. It is hypothesized that acid is more affective in the decreasing of hemicelluloses. Combined and enzymatic treatments appeared to be more effective in the removal of some lignin contents in comparison to acidic and non treated PPW.

## Percent Saccharification

Figure 1 illustrates the percent saccharification of PPW for different time intervals from 4 to 120 h under different hydrolysis strategies. Every category showed progression in percent saccharification for various incubation time periods. Maximum saccharification (21.23±0.13%**)** was observed with combined hydrolytic treatment with *Bacillus sp.* CG2 at 96 h, which manifested the efficient biomass breakdown and a synergistic effect between acidic pretreatment and *Bacillus sp.* CG2 enzymes.

Maximum saccharification of combined hydrolytic treatment with *Bacillus manliponensis* CA2 was 17.21±0.24% at 96 hours. *Bacillus manliponensis* CA2 showed effective hydrolysis but with slower pace than *Bacillus* sp. CG2CG2. This might be due to less substrate affinity and enzyme efficiency. The same trend was observed with enzymatic hydrolysis where *Bacillus sp.* CG2 showed higher peaks of percent saccharification than *B. manliponensis* CA2.

Acidic hydrolysis resulted in 13.73±0.22%. Percent saccharification peaks were observed at 96 h followed by decline up to 120 hours. This might be due to enzyme inactivation or substrate saturation. On the basis of highest saccharification rate, PPW hydrolyzate treated with the combined method using *Bacillus sp.* CG2 was selected for the fermentation experiment, as this treatment released the greatest amount of sugars.

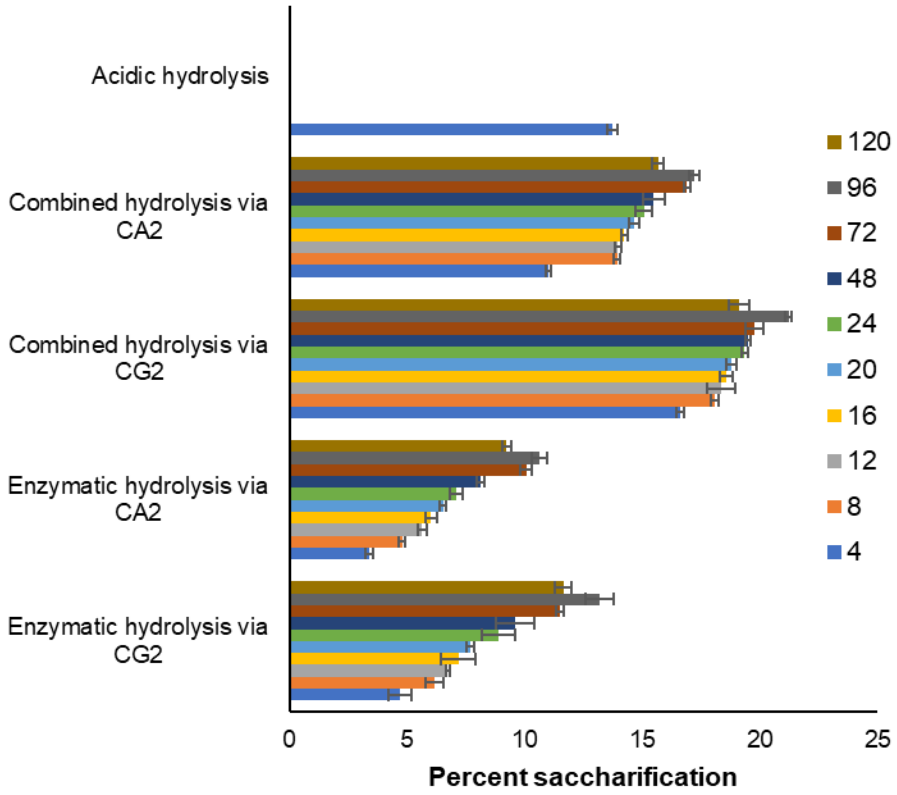


**Fig. 1.** Percent saccharification of PPW for different time interval employing acidic, enzymatic, and combined hydrolysis techniques *via Bacillus manliponensis* CA2 as well as *Bacillus sp.* CG2

## Detoxification of PPW hydrolyzate

Following treatment with sulfuric acid for acidic and combined processes, a 58% decrease in phenolic compounds was observed following detoxification with 2.5% activated charcoal. The concentration of phenolic compounds (mg/mL) in PPW hydrolysate prior to detoxification was 1.75 ±0.11, and this level was decreased to 1.03 ±0.17 afterwards.

## Bioethanol Titer

PPW hydrolyzates after different treatments were subjected to fermentation by two yeast isolates *viz.*, *Saccharomyces cerevisiae* K7 and *Metschnikowia cibodasensis* Y34 (Table 4). For the PPW hydrolyzates prepared through enzymatic and combined treatments, *Bacillus sp.* CG2 was selected. Slightly improved ethanol contents across all hydrolyzates were observed by *Metschnikowia cibodasensis* Y34, reaching their peaks from days 3 to 6. For about 10 days, bioethanol contents of all hydrolysate of PPW was measured on daily basis. At the 4$^{th}$ day, 15.00 ± 0.08 g/L ethanol contents were recorded. Under enzymatic treatment maximum ethanol titer was 14.62 ± 0.49 g/L at day 4. Maximum ethanol titer produced as a result of combined PPW hydrolysate fermentation at day 4 was 16.05 ± 0.66 g/L. The maximum estimation of ethanol contents by PPW hydrolysate from combined treatment provided rich fermentable sugar contents, enhancing ethanol fermentation efficiency. Prolonged fermentation is helpful to evaluate the tolerance of yeast isolates to accumulated ethanol and degradation of ethanol in medium. These observations are important for determining the optimal fermentation duration and ensuring process reliability for scale-up.

**Table 4.** Ethanol Titer (g/L) in Different Hydrolyzates of PPW with Combined-Acidic + Enzymatic

Data are presented as mean values of triplicates ± standard error of the mean (SEM). Within each column, values not sharing a common letter differ significantly ($p \leq 0.05$) based on one-way ANOVA.

| Incubation Days | Acidic | | Enzymatic | | Combined | |
|---|---|---|---|---|---|---|
| | K7 | Y34 | K7 | Y34 | K7 | Y34 |
| 1 | A,B 5.80± 0.49 | AB 11.51 ± 0.06 | BC 12.28 ± 0.39 | BC 12.55 ± 0.59 | BC 12.78 ± 0.47 | BC 13.58 ± 0.18 |
| 2 | A,B 7.15± 0.38 | A 12.77 ± 0.16 | ABC 12.75 ± 0.24 | AB 13.09 ± 0.17 | A 13.60 ± 0.75 | ABC 14.57 ± 0.43 |
| 3 | A,B 8.13± 0.10 | A 14.69 ± 0.60 | A 13.53 ± 0.05 | AB 13.58 ± 0.18 | A 13.71 ± 0.27 | ABC 14.99 ± 0.61 |
| 4 | A 10.01±0. 73 | A 15.00 ± 0.08 | A 13.64 ± 0.04 | A 14.62 ± 0.49 | A 14.01 ± 0.17 | AB 16.05 ± 0.66 |
| 5 | A,B 8.75± 0.05 | AB 14.61 ± 0.21 | AB 13.44 ± 0.06 | A 14.45 ± 0.11 | AB 13.87 ± 0.13 | A 15.76 ± 1.15 |
| 6 | A,B 6.69± 0.69 | AB 14.40 ± 0.04 | AB 13.38 ± 0.16 | AB 13.86 ± 0.03 | AB 13.76 ± 0.25 | ABC 15.39 ± 0.45 |
| 7 | A,B 6.46± 0.82 | AB 12.37 ± 0.11 | AB 13.29 ± 0.21 | AB 13.58 ± 0.18 | AB 12.76 ± 0.18 | ABC 14.44 ± 0.08 |
| 8 | A,B 5.63± 0.17 | AB 11.07 ± 0.37 | ABC 12.74 ± 0.51 | BC 12.66 ± 0.70 | BC 11.80 ± 0.30 | BC 13.84 ± 0.19 |
| 9 | B 5.04± 0.58 | AB 10.78 ± 0.13 | C 11.84 ± 0.19 | CD 11.24 ± 0.07 | C 10.74 ± 0.17 | BC 13.53 ± 0.29 |
| 10 | B 2.99± 0.35 | AB 09.24 ± 0.45 | C 11.60 ± 0.16 | D 10.51 ± 0.18 | C 10.59 ± 0.16 | C 13.28 ± 0.08 |

## Ethanol Yield

Table 5 depicts the ethanol yield in PPW hydrolyzates obtained after different treatments for 10 days. Maximum yield of ethanol was 0.39 g/g and 0.37 at day 3 with acidic hydrolyzate fermented with both yeast isolates K7 and Y34 correspondingly. Maximum ethanol yield at the 4$^{th}$ day was 0.39 g/g and 0.38 g/g as a result of enzymatic PPW hydrolyzate fermentation by *Metschnikowia cibodasensis* Y34 and *Saccharomyces cerevisiae* K7, respectively. In combined PPW hydrolyzate fermentation, ethanol yield (g/g) was enhanced gradually up to day 4 to maximum values that were 0.41 and 0.39 g/g by Y34 and K7, respectively. The improved yield was observed from day 3 to 5 that might be due to high sugar concentrations in fermentation medium and exponential growth phase of yeast cells.

**Table 5.** Ethanol Yield (g/g) in Different Hydrolyzates of PPW

| Incu-bation Days | Acidic | | Enzymatic | | Combined | |
|---|---|---|---|---|---|---|
| | K7 | Y34 | K7 | Y34 | K7 | Y34 |
| 1 | BC 0.29±0.09 | ABC 0.32±0.02 | AB 0.35±0.07 | AB 0.35±0.21 | AB 0.35±0.002 | B 0.31±0.11 |
| 2 | BC 0.34±0.10 | AB 0.35±0.01 | AB 0.36±0.08 | AB 0.36±0.03 | AB 0.36±0.17 | AB 0.34±0.11 |
| 3 | A 0.39±0.75 | A 0.37±0.05 | A 0.36±0.08 | AB 0.36±0.07 | AB 0.37±0.11 | AB 0.35±0.009 |
| 4 | AB 0.35±0.23 | AB 0.35±0.03 | A 0.38±0.11 | A 0.39±0.12 | A 0.39±0.02 | A 0.41±0.03 |
| 5 | AB 0.35±0.03 | ABC 0.34±0.18 | AB 0.37±0.001 | B 0.31±0.06 | AB 0.34±0.5 | B 0.31±0.31 |
| 6 | AB 0.33±0.08 | ABC 0.33±0.11 | AB 0.36±0.013 | C 0.23±0.13 | AB 0.34±0.03 | C 0.23±0.11 |
| 7 | BC 0.31±1.0 | ABC 0.30±0.09 | B 0.36±0.10 | C 0.21±0.12 | BC 0.30±0.14 | CD 0.20±0.001 |
| 8 | BC 0.29±0.05 | BCD 0.28±0.01 | C 0.37±0.02 | CD 0.18±0.031 | BC 0.29±0.21 | DE 0.14±0.022 |
| 9 | BC 0.29±0.04 | CD 0.27±0.02 | AB 0.35±0.2 | CD 0.17±0.02 | CD 0.25±0.10 | DE 0.13±0.01 |
| 10 | C 0.25±0.05 | D 0.21±0.01 | AB 0.31±0.04 | D 0.14±0.11 | D 0.19±0.02 | E 0.08±0.06 |

Data are presented as mean values of triplicates ± standard error of the mean (SEM). Within each column, values not sharing a common letter differ significantly ($p \leq 0.05$) based on one-way ANOVA.

## Fermentation Efficiency

Data in Table 6 revealed that *M. cibodasensis* Y34 performed better than *S. cerevisiae* K7 in terms of fermentation efficiency in all types of hydrolysates. Over 10 days, best fermentation efficiencies were noted from day 2 to 5. Species *M. cibodasensis* Y34 fermented PPW acidic, enzymatic, and combined hydrolyzates with percent efficiencies of 71.0, 78.7, and 83.8 respectively. The same trend was followed by standard yeast, though with a slow pace, from day 2 to 5. During enzymatic treatment, *S. cerevisiae* K7 demonstrated better fermentation efficiency than *M. cibodasensis* Y34 on day 4, despite the ethanol yield showing the reverse trend. This might be attributed to lower sugar consumption by Y34 due to limited sugar availability. This suggested that from day 2 to 5, both yeasts largely consumed fermentable sugars with high bioconversion rate, as depicted by ethanol yield. After day 4, yeast didn't maintain the high bioconversion rate, leading to a drop in fermentation efficiency.

**Table 6.** Fermentation Efficiency (%) of Yeast Isolates in Different Hydrolyzates of PPW

| Incu-bation Days | Acidic | | Enzymatic | | Combined | |
|---|---|---|---|---|---|---|
| | K7 | Y34 | K7 | Y34 | K7 | Y34 |
| 1 | E: 54.67±0.001 | E: 56.87±0.21 | E: 59.37±0.01 | D: 69.22±0.10 | D: 69.90±0.05 | D: 73.31±0.13 |
| 2 | C: 64.13±0.30 | C: 67.33±0.05 | B: 74.61±0.31 | B: 72.84±0.03 | C: 71.40±0.2 | B: 77.79±0.09 |
| 3 | B: 66.10±0.11 | B: 69.12±0.13 | C: 68.21±0.09 | C: 71.08±0.02 | B: 73.09±0.01 | C: 76.10±0.24 |
| 4 | A: 69.10±0.15 | A: 71.04±0.04 | A: 79.51±0.33 | A: 78.72±0.21 | A: 80.80±0.19 | A: 83.75±0.01 |
| 5 | D: 55.79±0.04 | D: 58.99±0.11 | D: 60.20±0.06 | E: 61.4±0.14 | C: 71.19±0.04 | E: 67.56±0.21 |
| 6 | F: 46.81±0.18 | F: 48.84±0.04 | F: 44.55±0.05 | F: 45.43±0.13 | E: 58.50±0.11 | F: 49.39±0.03 |
| 7 | G: 43.19±0.19 | G: 46.22±0.12 | G: 38.65±0.08 | G: 40.72±0.07 | F: 45.39±0.19 | G: 46.69±0.04 |
| 8 | H: 35.20±0.22 | H: 38.22±0.20 | H: 27.98±0.07 | H: 36.27±0.03 | G: 37.87±0.11 | H: 40.31±0.12 |
| 9 | I: 23.40±0.23 | I: 26.440±0.04 | I: 24.40±0.23 | I: 32.38±0.09 | H: 29.45±0.23 | I: 38.28±0.12 |
| 10 | J: 22.39±0.06 | J: 25.42±0.10 | J: 18.17±0.05 | J: 27.82±0.10 | I: 20.19±0.03 | J: 24.78±0.26 |

Data are presented as mean values of triplicates ± standard error of the mean (SEM). Within each column, values not sharing a common letter differ significantly ($p \leq 0.05$) based on one-way ANOVA.

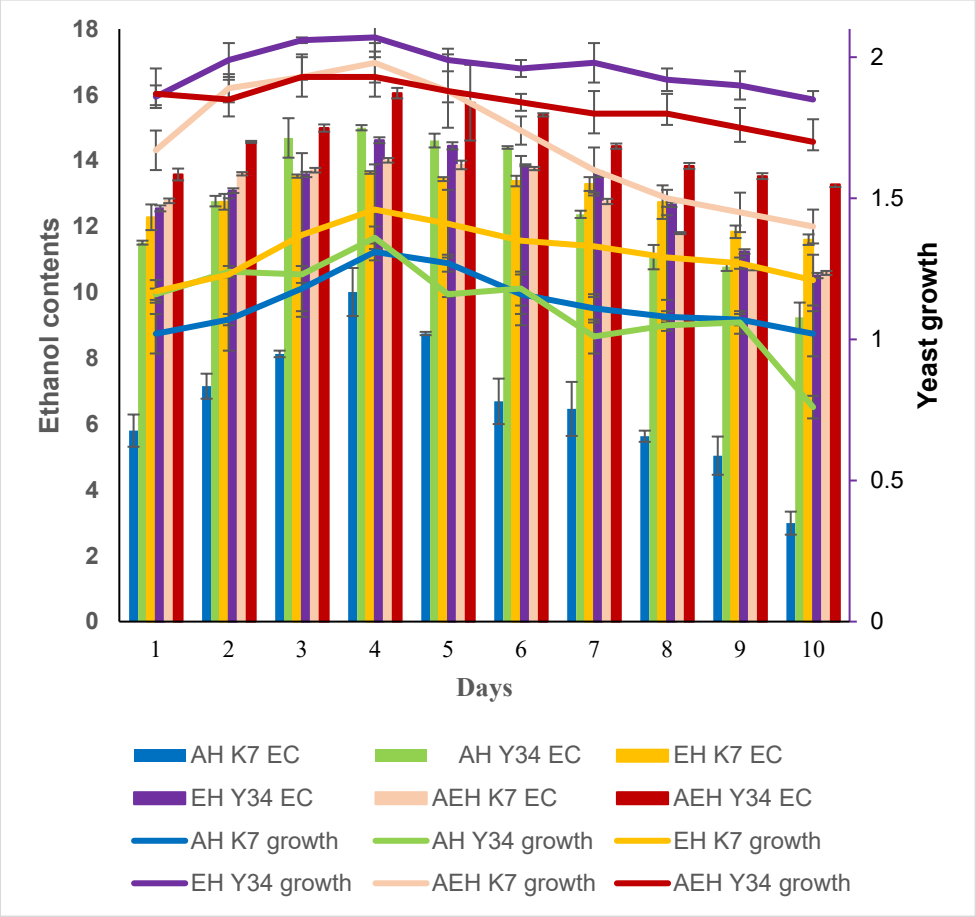


**Fig. 2.** Correlation between ethanol contents (g/L) in different hydrolyzates and yeast growth at 600 nm (AH-acidic hydrolyzate, EH-enzymatic hydrolyzate, AEH-Acidic+enzymatic hydrolyzate, K7- *S. cerevisiae* K7, Y34- *M. cibodasensis* Y34)

### Correlation between Ethanol Contents and Yeast Growth

Figure 2 shows positive interaction between ethanol contents (in bars) and yeast growth (lines) over 10 days across three different hydrolyzates. As yeast biomass increases (until day 4), ethanol production also increases. After day 4, both yeast growth and ethanol levels decline. Yeast growth followed a similar pattern to ethanol production as it increased until days 3 to 4 and then gradually declined. From graph it appeared that *S. cerevisiae* K7 exhibited a lower growth pattern than Y34 in all hydrolyzates indicating more biomass production.

## DISCUSSION

For microbial conversion of biomass, selection of an appropriate substrate is a critical step for efficient cellulase production. The substrate serves not only as a carbon and energy source but also provides essential nutrients to support microbial growth and enzyme secretion (Javed *et al*. 2019). The rapid increase in pomegranate consumption has led to a substantial rise in waste, particularly in the form of peels. Through hydrolysis/pre-treatment, reducing sugars can be effectively recovered from this lignocellulosic waste. The compositional analysis of pomegranate peel waste (PPW) was conducted using a range of established analytical protocols revealing hemicellulose 30.01±1.10 %, lignin 15.30±1.30 %, cellulose 32.62±0.40 %, reducing sugars 22.1 ± 0.12 g/L, and total sugars 79.6 ± 0.04 g/L. The findings are contrary to the values reported by Ibrahim (2013), which were approximately 10.8% hemicellulose, 5.7% lignin, 27.9% pectin, and 5.7% cellulose. Carbohydrate contents in this study was lower than the findings (66.31, 70.76 ±2.9 %) reported by Ranjitha *et al*. (2018) and Utami *et al*. (2025), respectively. These compositional variations might be due to different analytical methods, protocol selection, processing conditions, and geographic differences. In general, PPW is rich in complex polysaccharides, which makes it a potential feedstock for bioethanol production.

In this study, PPW was subjected to acidic, enzymatic, and combined (acidic + enzymatic) hydrolysis to assess reducing sugar release and its subsequent conversion into ethanol. Among the various pretreatment methods, dilute sulfuric acid is one of the most extensively studied due to its efficiency and cost-effectiveness compared to other acids such as nitric, hydrochloric, and phosphoric (Sarkar *et al*. 2012; Saleem *et al*. 2022). In this study, optimized conditions for acid hydrolysis were 6% $H_2SO_4$ at 50 °C for 60 min. Acidic pretreatment resulted in substantial sugar release, which is consistent with previous findings on corn stover and other lignocellulosic feedstocks (Jennings and Schell 2011; Unhasirikul *et al*. 2012; Adnan *et al*. 2014). Enzymatic pretreatment, recognized for its eco-friendly approach, has also gained significant attention (Maurya *et al*. 2015). Effective enzymatic saccharification requires accurate identification of lignocellulose-degrading microbes capable of producing cellulases, which play a pivotal role in converting cellulose to fermentable sugars. In this context, enzymatic conversion was investigated on PPW for up to five days, with periodic analysis of reducing and total sugars. Enzymatic hydrolysis by *Bacillus sp.* CG2 led to increase in sugar concentrations (53.29±2.20 g/L reducing sugar, and 119.26± 3.19 g/L total sugars). The maximum reducing sugars were observed after combined (acid + enzymatic) hydrolysis 85.77 ± 1.62 g/L, while total sugars reached 197.18 ± 10.38 g/L after 96 hours. The notably improved levels of reducing sugars observed after hydrolysis are consistent with findings reported in previous studies (Gomathi *et al*. 2012; Bhandari *et al*. 2013). An increase in reducing sugars (50% in dry

matter) was described by high voltage electrical discharges with enzymatic hydrolysis (El Kantar *et al*. 2018). In another study, 91.9% glucose yield was observed with wet-milled post-treatment and organic solvent combined pretreatment (Long *et al*. 2023). However, pretreatment in combination with enzymatic hydrolysis is inevitable for boosting conversion of sugar polymers to release fermentable sugars by raising the reducing sugar concentrations, which are crucial for biofuel production (Satari *et al*. 2019).

After 96 h of enzymatic and combined treatment with *Bacillus sp.* CG2, percent saccharification yield reached up to 13.19%, and 21.23%. Maximum saccharification in combined hydrolysis indicated a synergistic effect among the tested conditions in this investigation. This saccharification yield (13.19% and 21.23%) was low when compared with values generated from industrial application. In lignocellulosic biomass conversion, saccharification yields above 60 to 70% are commonly considered to ensure economic feasibility and efficient downstream fermentation (Alvira *et al*. 2013). Demiray *et al.* (2019) and Kumar *et al*. (2020) reported sugar yields 65% (pomegranate peel with dilute acid pretreatment) and 70% (citrus and fruit peel residues with combined physicochemical pretreatments). Similarly, enzymatic hydrolysis of agricultural wastes resulted in improved sugar yields (Josefsson 2013). The hydrolysis efficiency depends on several factors, including the lignin content, which acts as a barrier to enzyme accessibility. In the present study, lignin contents are low (15.30±1.30 %). But variations in lignin content, even with low range, can change the hydrolysis efficiency by altering substrate porosity, surface area, and enzyme–substrate interactions and might be the relevant factor to influence the hydrolysis efficiency in this study. Wheat bran, with lower lignin and higher hemicellulose content, showed higher saccharification yields compared to PPW and rice husk (Sakakibara *et al*. 2009). Similarly, yields between 55 and 75% have been reported for other fruit wastes such as banana peel and orange peel when appropriate delignification and hemicellulose solubilization strategies were applied (Saini and Kaur 2024). Similar two-fold increases in saccharification rates have been reported when using fungal isolates (Taha *et al*. 2015). Du *et al*. (2011) achieved an 82% hydrolysis yield using *Irpex lacteus* on corn stalks over 28 days, highlighting the potential of biological treatments. In the present study, *Metschnikowia cibodasensis* Y34 performed best in three hydrolyzates. The maximum ethanol production, 16.05 ± 0.66 g/L, was observed on day 4 in the combined (acid + enzymatic) hydrolysate, with a yield of 0.41 g/g and 83.75% fermentation efficiency. These findings are consistent with previous reports by Demiray *et al*. (2019), who achieved 13 g/L ethanol with 98% yield, under different conditions.

To enhance ethanol production, PPW hydrolysate was enriched with nutrients including peptone, yeast extract, $MgSO_4 \cdot 7H_2O$, $KH_2PO_4$, $CaCl_2$, and $ZnSO_4$. This was similar to the supplementation strategy adopted by Demiray *et al*. (2018), who reported increased ethanol production by *S. cerevisiae* up to 44.9%. In contrast, Göksungur and Zorlu (2001) reported a maximum ethanol yield of 4.62% from sugar beet molasses at high biomass loading, highlighting the impact of substrate concentration on yield. Fermentation using both *S. cerevisiae* and *Metschnikowia sp*. demonstrated that PPW can serve as a viable substrate for second-generation ethanol production. The integration of pretreatment, saccharification, and fermentation processes proved effective in improving overall ethanol yield. This study highlights the potential of PPW as a low-cost, renewable feedstock for sustainable bioethanol production, contributing to the advancement of circular bioeconomy and waste valorization.

## CONCLUSIONS

1. This study demonstrated the effective conversion of pomegranate peel waste into bioethanol using acidic, enzymatic, and combined hydrolysis approaches followed by fermentation.
2. Among different hydrolysis techniques tested, combined hydrolysis (acidic + enzymatic) proved to be the most efficient, resulting in the maximum sugar release and maximum ethanol yield of 0.41 g/g, with a fermentation efficiency of 83.8%.
3. The cellulolytic activity of *Bacillus sp.* CG2 contributed to enzymatic hydrolysis performance.
4. Fermentation by *Metschnikowia cibodasensis* Y34 in the combined hydrolysate yielded the ethanol concentration **(**16.05 ± 0.66 g/L**)**, underscoring its potential as an alternative fermenting organism.
5. Overall, the findings support the sustainable valorization of fruit waste such as pomegranate peels waste (PPW) for bioethanol production, contributing to both waste management and renewable energy generation**.**
6. These findings strongly support the integration of acidic pretreatment with targeted bacterial enzymatic hydrolysis for efficient biomass valorization.

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### Competing Interests

The authors declare that there are no conflicts of interest.

### Availability of Data and Material

All the data generated in this research work has been included in this manuscript.

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